

Development & Evaluation of Chlorophyll *a* Fluorescence as a bioanalytical tool for pollutant identification

A thesis submitted for the degree of Doctor of Philosophy by

Rachael Smith (B Sc. Hons)

2010

Department of Environmental Science
University of Technology, Sydney

*This thesis is dedicated to my grandfather,
Geoff Murphy (1928-2009).*

*As a child, my grandfather's love and respect for the land, the bush and the ocean
was a true inspiration for me to one day be able to do all that I can to help save our
beautiful Earth.*

*Grandad – I hope you're proud that I've finished university and can finally get to
work.*

CERTIFICATE OF AUTHORSHIP/ORIGINALITY

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Signed 

Rachael Smith (PhD Candidate)

ACKNOWLEDGEMENTS

Although I have signed my name to this thesis, there are many people who have contributed in one way or another along the road to its completion. Those people are recognized and acknowledged here.

Imparting great wisdom of photosynthesis and other scientific and non-scientific truths, my supervisor Peter Ralph is particularly acknowledged for overseeing and guiding my research through all its triumphs and pitfalls. With Peter's patience, dedication and belief in my abilities, thankfully the triumphs managed to prevail. I would also like to acknowledge the other members of the Aquatic Photosynthesis Group (APG) for their valued input particularly for seminar and conference presentations and assistance with laboratory work.

I would like to acknowledge my co-supervisor, Jochen Müller, particularly in the early stages of my candidature for giving me strong advice and to help steer me in the right direction. I would also like to acknowledge Jochen's team at EnTOX whose knowledge of toxicology and chemistry was of great value. Derek Eamus, my co-supervisor at UTS, is also acknowledged for imparting great wisdom in tackling a thesis and a PhD. I also acknowledge the very many other academics and staff from UTS who were of immense value in giving experimental and statistical help and advice.

Davy Wong is acknowledged for his assistance and development of the MSF software program used in this thesis. Without his knowledge of mathematics and statistics it would not have been possible for me to develop the fingerprinting method described in this thesis. I would also like to acknowledge Michael Manion for his role in preparing my thesis for submission as a patent application.

Particular mention goes to Cliff Seery, Christopher Rawson and Jennifer Whan for reading and editing my thesis, being great support, giving great advice and being wonderful friends when all seemed to be lost. I would also like to acknowledge Cliff for his dedication to late night sampling in the rain for samples that didn't quite make it into the final thesis.

I have learnt also that a thesis doesn't get done without the many friends and family around you to help as much as possible. I would like to acknowledge my parents for their financial support. I also would like to acknowledge my stepfather, Richard Barnes, for the Excel macro's that he wrote for me and the glasses of French champagne in the Whitsundays when I really needed a holiday. I would like to acknowledge my father for his support and giving up lengths of time to listen to my science ramblings. I need to acknowledge my mother and mentor, Kim Barnes, for so many aspects of this thesis. My mother has been such a great source of inspiration for so many years, not to mention the amount of hours of thesis reading and editing that she has put in to help get it to its finished state. Lastly, I would like to acknowledge the very patient and understanding Cameron Harris, who has been the solidarity in my life, who helped me get through the long hours, went through the ups and downs with me, and helped minimize the mental craziness...now we can finally get married!

ABSTRACT

There is potential to improve water quality monitoring programs by generating pollution data that better represents the aquatic ecosystem being monitored. By incorporating rapid and cost-effective bioanalytical methods into water quality monitoring programs, risk associated with unrepresentative data can be reduced by increasing the number of samples collected without incurring additional costs. The rapid and cost-effective toxin-identification method presented here is based on quantifying patterns of change in chlorophyll *a* fluorescence (fluorescence fingerprints) associated with a toxicants mode of action (MoA). Chlorophyll *a* fluorescence yield is influenced by environmental factors and can be used to identify stress caused by light, nutrient status and the presence of pollutants. When the functional state of the photosynthetic apparatus changes, the yield of fluorescence emission also changes, generating a chlorophyll *a* fluorescence response that has previously been thought to be unique based on a toxicants mode of action.

The toxin-identification method was developed as a bioanalytical system based on the chlorophyll *a* fluorescence responses of a microalgae (*Dunaliella tertiolecta*) to herbicide and nutrient impacts, measured using the Imaging-PAM fluorometer. The analysis of the fluorescence response was the novel method; a holistic approach was employed. Unlike previous approaches which measured one fluorescence parameter for toxicant identification, the method presented here assessed the temporal unity of change in energy dissipation, which was found to be unique depending on a chemical's mode of action (i.e. its physico-chemical properties and toxicokinetic relationship with the organism). The method was tested for two different uses: (1) as a non-specific biosensor able to identify herbicides (and their potency) in a water sample of unknown constituents, and (2) a method specific to the identification and potency of nutrients in a water sample.

Seven herbicides were examined totaling three different MoAs; PSII inhibitors (DCMU, Irgarol, Bromacil and Simazine), uncoupling of phosphorylation (Dinoseb and PCP) and creation of reactive oxygen species (paraquat). By first generating a database of reference response patterns, the response patterns of laboratory derived test samples were then measured and quantitatively compared to the reference

patterns. The unknown or test sample was compared to reference toxicants using a mean-square fit (MSF) software program. The MSF program tells the user how well the fingerprint of the test sample fits to each of the fingerprints of the reference chemicals. The method showed 93% accuracy in correctly identifying six herbicides, with false negative identifications occurring for only two toxicants, simazine (8% of samples) and Dinoseb (27% of samples).

Phosphate induced fluorescence transients were also assessed to demonstrate that the toxin-identification method was versatile in its ability to also be used as a selective biomarker. By culturing P-limited *D. tertiolecta* cells, a unique fluorescence response was recorded upon additions of PO_4^{3-} . The NIFT (nutrient induced fluorescent transient) response was specific to PO_4^{3-} additions compared to NH_4^{3+} and NO_2^- additions. Quantification of the NIFT response showed high levels of precision and specificity for multiple fluorescence parameters.

The toxin-identification method presented here is still in its preliminary stages and higher levels of validation are still necessary including testing environmental samples, and comparing results from the toxin-identification method to results from chemical analysis. However, this thesis presents the foundational work of a unique and powerful bioanalytical tool with the potential to greatly improve water quality management practices.

CONTENTS

CERTIFICATE OF AUTHORSHIP/ORIGINALITY	iii
ACKNOWLEDGEMENTS	iv
ABSTRACT	vi
CONTENTS	viii
LIST OF FIGURES	xii
LIST OF TABLES	xv
LIST OF ABBREVIATIONS	xvii
1.0 INTRODUCTION	2
1.1 Pollution Monitoring	2
1.2 Pollutant Identification	6
1.3 Chlorophyll <i>a</i> Fluorescence Toxicology	9
1.3.1. Status of chlorophyll <i>a</i> fluorescence in environmental toxicology	9
1.3.2. Photosynthesis and photoinhibition	11
1.3.3. Measurement of toxicant induced photoinhibition with chlorophyll <i>a</i> fluorescence	19
1.3.4. Fluorescence parameters and a toxicants mode of action	22
1.4 Aim of Thesis: Development of a chlorophyll <i>a</i> fluorescence fingerprint for toxicant identification and potency estimation	23
1.5 Thesis Outline	26
1.6 References	28
2.0 GENERAL METHODS	34
2.1 Introduction	34
2.2 Bioanalytical System	34
2.2.1. Test organism	34
2.2.2. Imaging-PAM fluorometer	36
2.2.2.1. Components and set-up of the Maxi Imaging-PAM fluorometer	37
2.2.2.2. System operation	38
2.2.3. Chlorophyll <i>a</i> fluorescence analysis	39
2.3 Operating Procedure	42
2.3.1. Batch culturing and test preparation	42
2.3.2. Culture calibration and standardization	42
2.3.3. Cell density determination	43
2.3.4. Test solutions	43
2.3.5. Solvent controls	43
2.3.6. Fluorescence measurements	45
2.4 Mathematical and Statistical Analysis	46
2.4.1. Computer software	46
2.4.2. Standard dose-response curves and effective concentrations	47

2.4.3.	Coefficient of variance and Variance	47
2.4.4.	Parallel curves and potency estimation	48
2.4.5.	Method for determining parallelism of dose-response curves and potency estimation using the MSF software program	48
2.5	References	54

3.0 OPTIMISATION OF CHLOROPHYLL *a* FLUORESCENCE TOXICITY TESTING FOR TOXICANT IDENTIFICATION USING THE IMAGING-PAM FLUOROMETER

58

3.1	Introduction	58
3.1.1.	Imaging-PAM fluorometer	58
3.1.2.	Multi-well plates	60
3.1.3.	Chapter aims	61
3.2	Materials and Methods	63
3.2.1.	Test chemicals	64
3.2.2.	Biomaterial	64
3.2.3.	Chlorophyll <i>a</i> fluorescence measurements	64
3.2.4.	Comparison of the light field within the wells of different types of 96-well plates	64
3.2.5.	Rapid light curves	65
3.2.6.	Test for optimal cell density	67
3.2.7.	Mathematical and statistical analysis	67
3.3	Results	69
3.3.1.	Stage 1: Heterogeneity of light field across 96-well plates	69
3.3.2.	Stage 2: Effect of well plate type on reflectance/attenuation of light	71
3.3.3.	Stage 3: Optimum cell density and Imaging-PAM setting selection for precision and sensitivity	75
3.4	Discussion	77
3.5	References	81
3.6	Appendix	83

4.0 TOXIN-IDENTIFICATION WITH CHLOROPHYLL *a* FLUORESCENCE

85

4.1	Introduction	85
4.1.1.	Reaction rates	87
4.1.2.	Mode of action and fluorescence parameters	88
4.1.3.	Parallelism of dose-response curves	91
4.1.4.	Chapter aims	92
4.2	Materials and Methods	94
4.2.1.	Test chemicals	94
4.2.2.	Biomaterial	94
4.2.3.	Cell density	94
4.2.4.	Chlorophyll <i>a</i> fluorescence measurements and analysis	94
4.2.5.	Exposure experiments	94
4.2.6.	Mathematical and statistical analysis	95
4.2.7.	Calculation of reference input data and acceptance range	96
4.2.8.	Calculation of parallelism	96
4.3	Results	100

4.3.1.	Reference curves	100
4.3.2.	Test sample curves	101
4.3.3.	Acceptance ranges	103
4.3.4.	Toxicant prediction	103
4.3.5.	Toxicant identification using multiple fluorescence parameters	106
4.3.6.	Time-dependent variations	108
4.4	Discussion	110
4.5	References	118
5.0	SINGLE POLLUTANT IDENTIFICATION FROM A MIXTURE	123
5.1	Introduction	123
5.1.1.	Mixture toxicology	123
5.1.2.	Effects of mixtures on chlorophyll <i>a</i> fluorescence	125
5.1.3.	Chapter aims	127
5.2	Materials and Methods	130
5.2.1.	Test chemicals	130
5.2.2.	Biomaterial	130
5.2.3.	Cell density	130
5.2.4.	Chlorophyll <i>a</i> fluorescence measurements and analysis	130
5.2.5.	Exposure experiments	130
5.2.6.	Mathematical and statistical analysis	132
5.3	Results	134
5.3.1.	Chemical interactions of binary mixtures	134
5.3.2.	Parallelism of binary mixtures to single toxicant data	137
5.4	Discussion	154
5.5	References	160
6.0	NUTRIENT INDUCED FLUORESCENCE TRANSIENTS (NIFTS): a SELECTIVE BIOMARKER FOR PO₄³⁻ ASSESSMENT	163
6.1	Introduction	163
6.1.1.	Physiological changes associated with nutrient limitation and Nutrient Induced Fluorescence Transients (NIFTs)	164
6.1.2.	Chapter aims	166
6.2	Materials and Methods	167
6.2.1.	Test chemicals	167
6.2.2.	Biomaterial	167
6.2.3.	Phosphate-limited and P-replete cultures	167
6.2.4.	Chlorophyll <i>a</i> fluorescence measurements and analysis	168
6.2.5.	Exposure experiments	168
6.2.6.	Mathematical and statistical analysis	169
6.3	Results	172
6.3.1.	Chlorophyll <i>a</i> fluorescence changes associated with PO ₄ ³⁻ limitation	172
6.3.2.	End-point validation	175
6.3.3.	Selectivity	178
6.3.4.	Effect of the presence of another pollutant on PO ₄ ³⁻ relative potency estimations	179
6.4	Discussion	184

6.5	References	189
7.0	DISCUSSION	193
7.1	Purpose of the Method	193
7.2	The Developed Method	194
7.3	Applying the Method: Types of Chemicals and Matrices	197
7.4	Standard Operating Procedure	200
7.5	Analytical Requirement: Evaluation of Method Performance Characteristics	204
7.6	Concluding Remarks	205
7.7	References	206

LIST OF FIGURES

Figure 1.1: Fluorescence induction or 'Kautsky' curve based on the PAM technique. A time course of changes in fluorescence upon illumination of ML (measuring light), SP (saturation pulse) and AL (actinic light) records the fluorescence parameters F_o (minimum fluorescence), F_m (maximum fluorescence), F_t (minimum fluorescence under photosynthetic active radiation) and F_m' (maximum fluorescence under photosynthetic active radiation).	20
Figure 1.2: Hierarchy of stages of the objectives and requirements for prevalidation, validation and standardization of new analytical methods. RSD=relative standard deviation; CV=coefficient of variation. Adapted from Taverniers, De Loose and Van Bockstaele (2004).	25
Figure 2.1: The Maxi-Imaging-PAM; (a) a schematic representation of the Maxi-Imaging-PAM (adapted from Walz 2009), (b) photographic picture showing the configuration of the LED lamps and CCD camera objective lens (Source: Walz 2009), and (c) a 2D image of chlorophyll fluorescence measured from a 96-well plate.	38
Figure 2.2: Changes in fluorescence of <i>Dunaliella tertiolecta</i> when spiked with controls (Milli-Q water) compared to solvent controls (NaOH + methanol). The x-axis indicates fluorescence changes of three fluorescence parameters; EQY, Y(NPQ) and Y(NO), at two exposure periods; 0.5 h and 2 h. Bars indicate average values \pm standard error of mean ($n = 66$ and 22 for controls and solvent controls, respectively). * indicates where a significant difference ($p \leq 0.05$) lies between Milli-Q and solvent controls.	45
Figure 2.3: Parallel shifts of a concentration-effect curve to the right along the x-axis (blue curve) and up along the y-axis (red curve).	50
Figure 2.4: Log dose-response curves of substances S1, S2 and S3 from Table 2.2.	52
Figure 2.5: Example of input data required for the MSF software program and the subsequent output data calculated from the MSF software program. Highlighted areas demonstrate the mean-square fit (or estimation of parallelism) between the S1 and S2 dose-response curves and the potency estimation of S2 compared to S1.	53
Figure 3.1: Flow diagram of experimental stages applied in this chapter in order to determine the settings and designations (i.e. plate type, plate testing region and cell density) to be used in the experimental methods for Chapters 4 – 6.	63
Figure 3.2: Divided regions of a 96-well plate; Region 1- middle 4 wells, Region 2- middle 16 wells, Region 3- middle 36 wells, Region 4- middle 48 wells and Region 5- all 96-wells.	69
Figure 3.3: Effect of <i>D. tertiolecta</i> cell density on EC50 concentrations based on the % change in EQY by DCMU (μM) tested with black 96-well plates (●) and Microtiter white plates + filter (○). Symbols and error bars represent mean \pm 1 S.D. ($n=3$).	76
Figure 3.4: Depiction of the light fields (reflection and absorption) within the MWP+F and BP wells measured by a spherical light sensor. Blue arrows indicate wavelengths of light emitted by the Imaging-PAM fluorometer.	79
Figure 3.5: Depiction of the light fields and chlorophyll a fluorescence of microalgal cells in the MWP+F and BP. Blue region represents light intensity emitted by the Imaging-PAM fluorometer, green dots indicate microalgae not emitting fluorescence and red dots indicate microalgae emitting fluorescence.	79
Figure 4.1: From left to right; chemical characteristics of toxicants impacting on the light reactions of the photosynthetic apparatus, their identifying characteristics (based on pharmacological (A), chlorophyll a fluorescence (B) and toxicological (C) methods, and the measurable responses that, when combined (D), will theoretically produce a unique fluorescence fingerprint.	87
Figure 4.2: Methodology for calculation of the reference input data and acceptance range for toxicant reference samples. This process accounts for data of only one fluorescence parameter for an individual toxicant. The process is repeated for each toxicant and each fluorescence parameter.	99
Figure 4.3: Methodology for calculation of parallelism of test samples with reference data of each toxicant. This process accounts for data of only one fluorescence parameter for an individual test sample. The process is repeated for each test sample and each fluorescence parameter.	99
Figure 4.4: Box-plot of R^2 values of EQY, Y(NPQ) and Y(NO) at 30 minutes and 2 h dose-response curves for (a) DCMU, (b) Irgarol 1051, (c) Bromacil, (d) Simazine, (e) PCP, (f) Dinoseb, and (g) Paraquat. Top, middle and bottom lines of box indicate the 75 th percentile, median and 25 th percentile, error bars above and below the box indicate the 90 th and 10 th percentiles ($n=12$).	102
Figure 4.5: Dose-response changes in EQY of <i>D. tertiolecta</i> exposed to the PSII inhibitors; DCMU, Simazine, Irgarol 1051 and Bromacil for 30 minutes (●) and 2 h (○). Values represent mean % change in EQY from control (\pm 1 S.D.), $n=12$	109

Figure 5.1: Isobologram (adapted from Altenburger et al. 1993) illustrating three types of joint action of a binary mixture: additive, antagonistic and synergistic. Isoboles are lines representing a defined effect level and indicate the mixture ratios of the two substances (S1 and S2) required to achieve that effect level. The straight line indicates additivity, an upward-bent line indicates antagonism and a downward-bent line indicates synergism.	129
Figure 5.2: Experimental design of a 6-6 dose-combination matrix for a binary mixture of substances 1 and 2 at various concentrations (C_0, \dots, C_6) (adapted from Altenburger et al. 2003). Circles represent dose-combinations. Green horizontal lines represent an n.n experimental design where various concentrations of Substance 1 (S1) as the primary toxicant are tested with a fixed concentration of Substance 2 (S2) as the secondary toxicant: S1(1°) + S2 (2°). Yellow lines represent the converse, i.e. the dose-combinations when S2 is the primary toxicant with fixed concentrations of S1 as the secondary toxicant: S2(1°) + S1(2°). Dose-combinations for the ray experimental design are represented by red circles. Black lines represent single toxicant reference data for S1 (horizontal line) and S2 (vertical line).	132
Figure 5.3: Isobolic representations of binary mixture dose-response relationships illustrating (a) concentration addition, (b) antagonism, and (c) dose level-dependent deviation. Lines and values indicate effect concentrations, S1 and S2 are substances 1 and 2 of the binary mixture (adapted from Jonker et al. 2005).	133
Figure 5.4: Isobolic representation of the % mean change in (a) EQY, and (b) Y(NO) for the 6-6 matrix of DCMU + Bromacil dose combinations; values indicate response levels (n=4).	135
Figure 5.5: Isobolic representation of the % mean change in (a) EQY, and (b) Y(NPQ) for the 6-6 matrix of Dinoseb + PCP dose combinations; values indicate response levels (n=4).	135
Figure 5.6: Isobolic representation of the % mean change in (a) EQY, (b) Y(NO), and (c) Y(NPQ) using a 6-6 matrix of DCMU + PCP dose combinations; values indicate response levels (n=4).	137
Figure 5.7: Average mean-square fits (n = 4) of primary toxicant dose-response curves with increasing additions of the secondary toxicant fitted to both the primary and secondary toxicant reference data. Figures on the left represent DCMU (●) as the primary toxicant with additions of Bromacil (●) as secondary toxicant for the fluorescence parameters (a) EQY and (c) Y(NO). Figures on the right represent Bromacil (●) as primary toxicant and DCMU (●) as secondary toxicant for the fluorescence parameters (b) EQY and (d) Y(NO). Dotted lines represent acceptance range derived from single toxicant reference data for DCMU (black) and Bromacil (red). Solid lines represent regression lines for DCMU data (black) and Bromacil data (red).	139
Figure 5.8: Changes in EQY of DCMU + Bromacil mixtures based on the n.n experimental design: (a) DCMU (1°) + Bromacil (2°), 0.25 and 1.0 µM Bromacil, compared to DCMU (red line) and Bromacil (inset) single toxicant reference curves; and (b) Bromacil (1°) + DCMU (2°), 0.02 and 0.04 µM DCMU, compared to Bromacil (red line) and DCMU (inset) single toxicant reference curves. Values represent mean ± 1 S.D. (n = 4).	141
Figure 5.9: DCMU:Bromacil mixture dose-response curves (●) based on the ray design, and DCMU (▼) and Bromacil (■) single toxicant reference curves for the fluorescence parameters (a) EQY and (b) Y(NO). Values represent mean ± 1 S.D. (n = 4).	142
Figure 5.10: Average mean-square fits (n = 4) of primary toxicant dose-response curves with increasing additions of the secondary toxicant fitted to both the primary and secondary toxicant reference data. Figures on the left represent Dinoseb (●) as the primary toxicant with additions of PCP (●) as secondary toxicant for the fluorescence parameters (a) EQY and (c) Y(NO). Figures on the right represent PCP (●) as primary toxicant and Dinoseb (●) as secondary toxicant for the fluorescence parameters (b) EQY and (d) Y(NO). Dotted lines represent acceptance range derived from single toxicant reference data for Dinoseb (black) and PCP (red). Solid lines represent regression lines for Dinoseb data (black) and PCP data (red).	144
Figure 5.11: Changes in EQY of Dinoseb + PCP mixtures based on the n.n experimental design: (a) Dinoseb (1°) + PCP (2°), 10 and 25 µM PCP, compared to Dinoseb (red line) and PCP (inset) single toxicant reference curves; and (b) PCP (1°) + Dinoseb (2°), 0.02 and 0.04 µM Dinoseb, compared to PCP (red line) and Dinoseb (inset) single toxicant reference curves. Values represent mean ± 1 S.D. (n = 4).	146
Figure 5.12: Dinoseb:PCP mixture dose-response curves (●) based on the ray design, and Dinoseb (▼) and PCP (■) single toxicant reference curves for the fluorescence parameters (a) EQY and (b) Y(NPQ). Values represent mean ± 1 S.D. (n = 4).	147
Figure 5.13: Average mean-square fits (n = 4) of primary toxicant dose-response curves with increasing additions of the secondary toxicant fitted to both the primary and secondary toxicant reference data. Figures on the left represent DCMU (●) as the primary toxicant with additions of PCP (●) as secondary toxicant for the fluorescence parameters (a) EQY and (c) Y(NO). Figures on the right	

represent PCP (●) as primary toxicant and DCMU (●) as secondary toxicant for the fluorescence parameters (b) EQY and (d) Y(NPQ). Dotted lines represent acceptance range derived from single toxicant reference data for DCMU (black) and PCP (red). Solid lines represent regression lines for DCMU data (black) and PCP data (red). 149

Figure 5.14: Changes in Y(NO) and Y(NPQ) of DCMU + PCP mixtures based on the n.n experimental design: (a) change in Y(NO) of DCMU (1 %) + PCP (2 %), 10 and 50 µM PCP, compared to DCMU (red line) and PCP (inset) single toxicant reference curves; and, (b) change in Y(NPQ) of PCP (1 %) + DCMU (2 %), 0.02 and 0.08 µM DCMU, compared to PCP (red line) and DCMU (inset) single toxicant reference curves. Values represent mean ± 1 S.D. (n = 4). 151

Figure 5.15: DCMU:PCP mixture dose-response curves (●) based on the ray design, and DCMU (▼) and PCP (■) single toxicant reference curves for the fluorescence parameters (a) EQY, (b) Y(NO) and (c) Y(NPQ). Values represent mean ± 1 S.D. (n = 4). 153

Figure 6.1: A single NIFT response (Fm') of P-limited *D. tertiolecta* to PO_4^{3-} (8 µM). Lower case letters indicate points at which descriptive curve variables are calculated (see Table 6.1). 170

Figure 6.2: Fluorescence yields, EQY, Y(NO) and Y(NPQ), of *D. tertiolecta* cultured in P-replete and P-limited growth media, (n = 9, ± 1 S.D.). * indicates significant differences (p ≤ 0.05) between P-replete and P-deplete fluorescence yields for individual fluorescence parameters. 173

Figure 6.3: Representative set of NIFT responses of P-limited *D. tertiolecta* to a range of PO_4^{3-} concentrations (0.5 – 8.0 µM) for the fluorescence parameters: Fm', Ft, EQY, Y(NO), Y(NPQ) and NPQ/4. 174

Figure 6.4: Representative set of NIFT responses of P-limited *D. tertiolecta* to (a) DCMU (0.01 µM), (b) PO_4^{3-} (0.1 µM) and (c) PO_4^{3-} (0.1 µM) + DCMU (0.01 µM) for the fluorescence parameters: Ft, Fm', Y(NO) and NPQ/4. 180

Figure 6.5: Representative set of NIFT responses of P-limited *D. tertiolecta* to (a) Cu^{2+} (10 µM), (b) PO_4^{3-} (0.1 µM) and (c) PO_4^{3-} (0.1 µM) + Cu^{2+} (10 µM) for the fluorescence parameters: Ft, Fm', Y(NO) and NPQ/4. 182

Figure 7.1: Cog graphs representing the levels of measurement of the test procedure that (a) were used in this thesis and (b) theoretically could be used to account for a greater range of toxicants. Cogs represent (from bottom to top): concentrations of the chemical/sample, fluorescence parameters, exposure period, light intensity and test species. 201

Figure 7.2: 3D fingerprints of data generated from experiments in Chapter 4. Both fingerprints show the % changes in EQY (colour code) over time for a series of seven dilutions. The fingerprint for DCMU (left) shows clear differences in the fingerprint for PCP (right). 203

LIST OF TABLES

Table 1.1: List of chemicals known to impact on the photosynthetic apparatus, mode of action and chlorophyll a fluorescence response..... 16

Table 2.1: Test chemicals and concentrations of the solvents used in stock solutions..... 44

Table 2.2: Serial dilutions and effect of two substances, S1, S2 and S3..... 51

Table 3.1: Variance of actinic light intensity supplied by the Imaging-PAM fluorometer across wells of four types of 96-well plates (± 1 S.D., $n=3$). Regions of wells are based on Figure 3.1..... 70

Table 3.2: Percent change in light intensity recorded within the middle four wells of four different 96-well plate types. Nineteen AL levels were tested and compared to the actual LED actinic light intensity of the Imaging-PAM. Values represent the mean % change (± 1 S.D.) of light intensities within wells from the actual LED actinic light intensities ($n = 4$). 71

Table 3.3: Minimum saturating irradiance (I_k) values were calculated from rapid light curves of *D. tertiolecta* tested in four different types of 96-well plates. Values represent the mean (± 1 S.D.) LED actinic light intensity of the Imaging-PAM fluorometer at which rETR is reached ($n=3$). 72

Table 3.4: Cell densities of *Dunaliella tertiolecta* and Imaging-PAM settings for the black 96-well plate and Microtiter white plate + filter required to to obtain an Ft reading of ~ 0.15 75

Table 3.5: Coefficients of variation (%) of *D. tertiolecta* DCMU (1 - EC50) concentrations (μM) at different cell densities tested in black 96-well plates and Microtiter white plates + filter..... 77

Table 3.6: Multi-well plate and Imaging-PAM specifications for use in the toxicant identification bioassay..... 80

Table 4.1: Physico-chemical properties of test chemicals and their mode of action (MoA). 98

Table 4.2: R^2 values of reference data to a 4-parameter sigmoid regression model ($n=12$). 100

Table 4.3: Acceptance ranges of toxicant reference data for the fluorescence parameters EQY, Y(NPQ) and Y(NO). Values represent average mean-square fit (± 1 S.D.), $n = 12$, n/a indicate where reference data did not fit the 4-parameter sigmoid regression model (see Table 4.2). 103

Table 4.4: Identification of toxicant test samples using parallelism of dose response curves for the fluorescence parameter EQY after a 30 minute exposure. Values are expressed as % ($n=12$), values in bold indicate a correct positive match, values either side of these are false positive errors. Acceptance range= Mean + 1 S.D. 104

Table 4.5: Identification of toxicant test samples using parallelism of dose response curves for the fluorescence parameter EQY after a 2 hour exposure. Values are expressed as % ($n=12$), values in bold indicate a correct positive match, values either side of these are false positive errors. Acceptance range= Mean + 1 S.D. 105

Table 4.6: Identification of toxicant test samples using parallelism of dose response curves for the fluorescence parameter Y(NPQ) after a 30 minute and 2 hour exposure. Values are expressed as % ($n=12$), values in bold indicate a correct positive match, values either side of these are false positive errors. Acceptance range= Mean + 1 S.D. 105

Table 4.7: Identification of toxicant test samples using parallelism of dose response curves for the fluorescence parameter Y(NO) after a 30 minute exposure. Values are expressed as % ($n=12$), values in bold indicate a correct positive match, values either side of these are false positive errors. Acceptance range= Mean + 1 S.D. 105

Table 4.8: Identification of toxicant test samples using parallelism of dose response curves for the fluorescence parameter Y(NO) after a 2 hour exposure. Values are expressed as % ($n=12$), values in bold indicate a correct positive match, values either side of these are false positive errors. Acceptance range= Mean + 1 S.D. 106

Table 4.9: Identification of toxicant test samples using composite response analysis based on the parallelism of dose response curves with an acceptance range of Mean + 1 S.D. for the fluorescence parameters, EQY, Y(NPQ) and Y(NO) after a 30 minutes and 2 hour exposure. Values are expressed as % ($n=12$), values in bold indicate a correct positive match, values either side of these are false positive errors..... 107

Table 4.10: Identification of toxicant test samples using a composite response analysis based on the parallelism of dose response curves with an acceptance range of Mean + 2 S.D. for the fluorescence parameters, EQY, Y(NPQ) and Y(NO) after a 30 minutes and 2 hour exposure. Values are expressed as % ($n=12$), values in bold indicate a correct positive match, values either side of these are false positive errors..... 107

Table 4.11: Percent of false negative predictions for the fluorescence parameter EQY at 30 minutes and 2 h exposures and the composite response analysis with acceptance ranges of mean + 1 S.D. and mean + 2 S.D., $n = 12$ 108

Table 4.12: Mean EC30 values (± 1 S.D.) calculated from DCMU, Simazine, Irgarol 1051 and Bromacil dose-response curves after 30 minute and 2 h exposures to <i>D. tertiolecta</i> .	109
Table 5.1: Mean-square values for DCMU:Bromacil mixture dilution curve fitted to DCMU and Bromacil reference curves for the fluorescence parameters EQY and Y(NO), and test for parallelism.	143
Table 5.2: Average (± 1 S.D., $n=4$) mean-square values for Dinoseb:PCP mixture dose-response curve fitted to Dinoseb and PCP single toxicant reference curves for the fluorescence parameters EQY and Y(NPQ), and test for parallelism.	148
Table 5.3: Mean-square values for DCMU:PCP mixture dilution curve fitted to DCMU and PCP reference curves for the fluorescence parameters EQY, Y(NPQ) and Y(NO), and test for parallelism.	153
Table 6.1: Descriptive curve variables of a NIFT response of P-limited <i>D. tertiolecta</i> to PO_4^{3-} , calculated from points on the curve (a, b, c, d) depicted in Figure 6.1.	170
Table 6.2: Average PO_4^{3-} potency estimates of PO_4^{3-} reference samples fitted to a PO_4^{3-} reference standard ($n = 3$).	176
Table 6.3: Coefficient of Variation of PO_4^{3-} potency estimates presented in Table 6.2	176
Table 6.4: Acceptance statistic (%) of PO_4^{3-} test dose-response data with a parallel fit to the PO_4^{3-} reference standard.	177
Table 6.5: Average PO_4^{3-} potency estimates of PO_4^{3-} test samples fitted to a PO_4^{3-} reference standard ($n=6$).	177
Table 6.6: Coefficient of Variation of PO_4^{3-} potency estimates presented in Table 6.5.	178
Table 6.7: Significant differences ($p \geq 0.05$) of the maximum change in Ft and Fm' for P-limited <i>D. tertiolecta</i> cultures with additions of PO_4^{3-} , NH_4^+ and NO_3^{2-} , and for P-replete <i>D. tertiolecta</i> cultures with PO_4^{3-} additions compared to Milli-Q control data.	178
Table 6.8: Average relative potency estimation and acceptance statistic of PO_4^{3-} + DCMU mixtures dose-response data fitted to a PO_4^{3-} reference standard ($n=3$). P-values of paired samples t-test ($df = 1$) of potency estimates of PO_4^{3-} /DCMU mixtures compared to PO_4^{3-} controls.	181
Table 6.9: Average relative potency estimation and parallel acceptance statistic of PO_4^{3-} /Cu ²⁺ mixtures dose-response data fitted to a PO_4^{3-} reference standard ($n=3$). P-values of paired samples t-test ($df = 1$) of potency estimates of PO_4^{3-} /Cu ²⁺ mixtures compared to PO_4^{3-} controls.	183

LIST OF ABBREVIATIONS

ΔF_{\max}	Maximum change in fluorescence
α_{\max}	Slope to the maximum change in fluorescence
AICS	Australian Inventory of Chemical Substances
AL	Actinic Light
ALi	Actinic Light intensity
ALw	Actinic Light width
ANOVA	Analysis of Variance
ANZECC	Australian and New Zealand Environment and Conservation Council
APHA	American Public Health Association
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatase
AUC	Area under the whole curve
AUC_{\max}	Integrated area under the curve from time 0 to time at ΔF_{\max}
BP	Black Plate
CA	Concentration Addition
CCD	Charge-Coupled Device
CITB	Chemical Information and Testing Branch
CoV	Coefficient of Variation
CSIRO	Commonwealth Scientific and Industrial Research Organisation
D1	D1 protein
EC	Effective Concentrations
EQY	Effective Quantum Yield of PSII
EU	European Union
Fm	Maximum Fluorescence (dark)
Fm'	Maximum Fluorescence under photosynthetic active radiation
Fo	Minimum Fluorescence (dark)
Fo'	Minimal fluorescence yield of an illuminated sample, lowered with respect to Fo by non-photochemical quenching
Ft	Fluorescence yield determined under photosynthetic active radiation

Fv/Fm	Maximum quantum yield
GCMS	Gas Chromatography Mass Spectrometry
HPLC	High Performance Liquid Chromatography
I_k	Minimum actinic light level at which the maximum rate of rETR (rETR _{max}) occurs
ISO	International Standardization Organization
LCMS	Liquid Chromatography Mass Spectrometry
LED	Light-Emitting Diode
LoD	Limit of Detection
Log K_{ow}	Octanol/water partitioning coefficient
LoQ	Limit of Quantitation
ML	Measuring Light
MLf	Measuring Light frequency
MLi	Measuring Light intensity
MoA	Mode of Action
MSF	Mean-Square Fit
MWP	Microtiter Microfluor (Thermo Scientific) 96-well Plates
MWP+F	Microtiter Microfluor (Thermo Scientific) 96-well Plates with Filter
N-limited	Nitrogen limited
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NIFT	Nutrient Induced Fluorescent Transient
NPQ	Non-Photochemical Quenching
P-limited	Phosphorous limited
PAH	Polycyclic Aromatic Hydrocarbon
PAM	Pulse Amplitude Modulated
PAR	Photosynthetically Active Radiation
Pi	Inorganic phosphorous
pK_a	Ionisation constant
PSI	Photosystem I
PSII	Photosystem II
qL	Coefficient of photochemical quenching (based on ‘lake’ model)

q_N	Coefficient of non-photochemical quenching
q_P	Coefficient of photochemical quenching (based on ‘puddle’ model)
Q_A	Plastiquinone A
Q_B	Plastiquinone B
R²	Coefficient of determination
rETR	Relative Electron Transport Rate
rETR_{max}	Maximum Rate of rETR
RLC	Rapid Light Curves
ROS	Reactive Oxygen Species
RSD	Relative Standard Deviation
SD	Standard Deviation
SNR	Signal to Noise Ratio
SOP	Standard Operating Procedure
SP	Saturation Pulse
SP_i	Saturation Pulse intensity
SPE	Solid Phase Extraction
TIE	Toxicity Identification Evaluation
USEPA	United States Environmental Protection Authority
WFD	Water Framework Directive
WP	White Plate
Y(NO)	Non-regulated non-photochemical quenching
Y(NPQ)	Regulated non-photochemical quenching